Dissection and modulation of the four distinct activities of nisin by mutagenesis of rings A and B and by C-terminal truncation

Rink, Rick; Wierenga, Jenny; Kuipers, Anneke; Kluskens, Leon D.; Driessen, Arnold J. M.; Kuipers, Oscar P.; Moll, Gert N.

Published in:
Applied Environmental Microbiology

DOI:
10.1128/AEM.01104-07

Applied and Environmental Microbiology, Sept. 2007, p. 5809–5816 Vol. 73, No. 18
0099-2240/07/$08.00+0 doi:10.1128/AEM.01104-07

Dissection and Modulation of the Four Distinct Activities of Nisin by Mutagenesis of Rings A and B and by C-Terminal Truncation▽†

Rick Rink,[1] Jenny Wierenga,[1] Anneke Kuipers,[1] Leon D. Kluskens,[1] Arnold J. M. Driessen,[2] Oscar P. Kuipers,[3] and Gert N. Moll[1]*

BiOMaDe Technology Foundation, Groningen, The Netherlands,[1] and Department of Microbiology[2] and Department of Molecular Genetics,[3] Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

Received 17 May 2007/Accepted 21 July 2007

Nisin A is a pentacyclic antibiotic peptide produced by various *Lactococcus lactis* strains. Nisin displays four different activities: (i) it autoinduces its own synthesis; (ii) it inhibits the growth of target bacteria by membrane pore formation; (iii) it inhibits bacterial growth by interfering with cell wall synthesis; and, in addition, (iv) it inhibits the outgrowth of spores. Here we investigate the structural requirements and relevance of the N-terminal thioether rings of nisin by randomization of the ring A and B positions. The data demonstrate that: (i) mutation of ring A results in variants with enhanced activity and a modulated spectrum of target cells; (ii) for the cell growth-inhibiting activity of nisin, ring A is rather promiscuous with respect to its amino acid composition, whereas the bulky amino acid residues in ring B abolish antimicrobial activity; (iii) C-terminally truncated nisin A mutants lacking rings D and E retain significant antimicrobial activity but are unable to permeabilize the target membrane; (iv) the dehydroalanine in ring A is not essential for the inhibition of the outgrowth of *Bacillus* cells; (v) some ring A mutants have significant antimicrobial activities but have decreased autoinducing activities; (vi) the opening of ring B eliminates antimicrobial activity while retaining autoinducing activity; and (vii) some ring A mutants escape the nisin immune system(s) and are toxic to the nisin-producing strain NZ9700. These data demonstrate that the various activities of nisin can be engineered independently and provide a basis for the design and synthesis of tailor-made analogs with desired activities.

Lantibiotics are (methyl)lanthionine-containing antibiotics (1, 9) produced by some gram-positive bacteria. The lanthionines are posttranslationally formed by enzyme-mediated dehydration of serine and threonine residues followed by enzyme-catalyzed intramolecular coupling to cysteines to form a thioether bridge. The lantibiotic nisin contains one lanthionine and four methyllanthionines (Fig. 1). Nisin autoregulates its own synthesis (19) by binding to the transmembrane protein NisK, which phosphorylates the intracellular response regulator NisR. Phosphorylated NisR activates the *nis* promoter.

Nisin predominantly acts against gram-positive bacteria. This occurs at nanomolar concentrations as a consequence of the interaction of nisin with the docking molecule lipid II (4, 6). Rings A and B physically interact with lipid II, and this results in membrane permeabilization by hybrid pores of nisin and lipid II (5) and inhibition of cell wall synthesis via lipid II abduction (11). Nisin-producing bacteria are protected against nisin by two self-protection mechanisms: a lipoprotein, NisI, which likely binds and inactivates nisin, and an export system, NisEFG, which presumably extrudes nisin from the cell (20). Another antibiotic activity of nisin is the inhibition of the outgrowth of spores. Nisin's dehydroalanine in position 5 has been reported to be involved in this inhibitory activity (29). Replacement of the dehydroalanine with an alanine at position 5 of nisin (7) and subtilin (27) strongly reduced the capacity to prevent the outgrowth of spores.

An alignment of lantibiotic sequences revealed a large group of nisin-resembling lantibiotics (32) that share the N-terminal rings A and B, ring A being composed of a lanthionine and three other amino acids and ring B being composed of a (methyl)lanthionine and two other amino acids. While the amino acid composition of ring A appears variable, ring B is rather conserved. The opening of ring A strongly reduces antimicrobial activity against *Micrococcus luteus* NCDO 8166 and leads to a complete loss of antimicrobial activity against *Lactococcus lactis* MG1614 (8). Only a few conservative ring A mutants (S3T, which has reduced activity [21]; S5A [7]; S5T [22]; and S5C [39]) and chemically modified variants (33) have been described, whereas no reports on ring B mutants have appeared. Here, we randomized the amino acids of these functionally important rings to investigate the possibility of engineering nisin mutants with improved or altered characteristics. Surprisingly, a large number of mutants with modulated activities were obtained, providing detailed information about the structural requirements of the different activities of nisin.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids are listed in Table 1.

Molecular cloning. Constructs coding for mutant prenisin were made by amplifying plasmid pNZnisA-E3 (18) using a phosphorylated downstream sense primer and an upstream antisense primer with a (nonannealing) peptide-encoding tail. The DNA amplification was carried out by using Phusion DNA polymerase (Finnzymes, Finland). The replacement of ISL of ring A of nisin involved primers 5′-NNNNNNNNNACTTGTAATGCGTGGTGATGC-3′ and 5′-PO_4-TGTACACCCGGTTGTAAAAG-3′, in which N is a randomized nucleotide. The replacement of PG of ring B with XX in nisin involved primers 5′-NNNNNNT

* Corresponding author. Mailing address: BiOMaDe Technology Foundation, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Phone: 31 50 363 80 70. Fax: 31 50 363 44 29. E-mail: Moll@biomade.nl.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 27 July 2007.

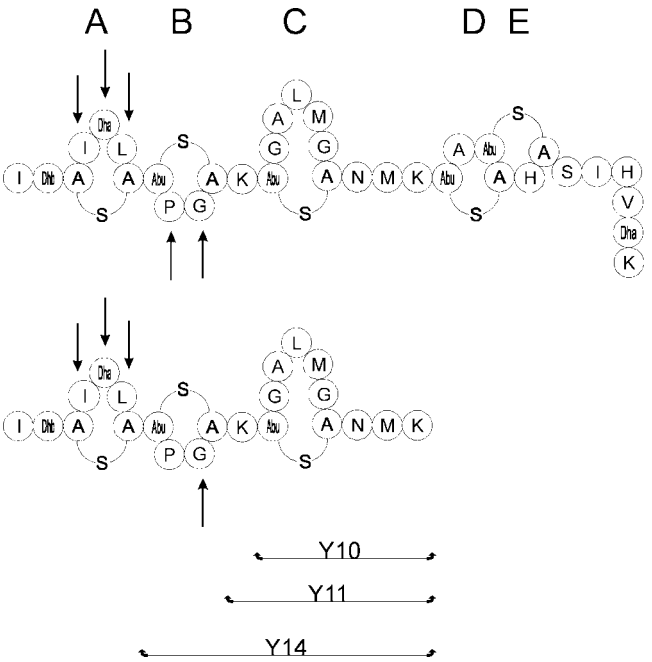

FIG. 1. Nisin A and nisin A(Δ23–34). The letters above the diagrams identify the rings. The arrows in the diagram of nisin A (top) indicate positions that have been randomized in this work. The arrows in the diagram of truncated nisin A (bottom) indicate mutated positions in mutants selected for truncation. Postsource decay fragments y10, y11, and y14 are depicted.

GTACATAGCGAAATACTTG-3′ and 5′-PO_4-TGTAAAACAGGAGCTCTG ATGG-3′. The replacement of PG with PX in ring B of nisin involved primer 5′-NNNGGGTGTACATAGCGAAATACTTG-3′. Self-ligation of the resulting plasmid encoding linear leader peptide was carried out with T4 DNA ligase (Roche). The electrotransformation of *L. lactis* was carried out as previously described (14) using a Bio-Rad gene pulser (Bio-Rad, Richmond, CA). Nucleotide sequence analysis was performed by BaseClear (Leiden, The Netherlands). Transformation mixtures of the sequenced transformants were directly plated out with or without 0.1 nM nisin in the agar, which contained trypsin to cleave off the leader. After overnight incubation, the transformants were overlaid with top agar containing the indicator strain *Lactococcus lactis* LL108(pORI80).

Culturing. *L. lactis* was grown in M17 broth (36) supplemented with 0.5% glucose (GM17) or minimal medium (32) with or without chloramphenicol (5 μg/ml) and/or erythromycin (5 μg/ml). The concentration of the antibiotics was reduced to 4 μg/ml when both selection markers were present simultaneously. *Staphylococcus epidermidis* K7 was grown on basic medium (30), *Lactobacillus johnsonii*, *Pediococcus pentosaceus*, and *Leuconostoc mesenteroides* on MRS medium, and *Micrococcus flavus* NIZO B423 on TY medium (Bacto tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter), all at 30°C; *Streptococcus thermophilus* was grown in GM17 medium at 42°C. Prior to mass spectrometric analyses of peptides produced in the media, cells were cultured as follows. Overnight cultures of *L. lactis* NZ9000 Δ*acmA* were grown in GM17 broth containing antibiotic(s) and were diluted 1/50 in minimal medium buffered with 0.12 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.0) supplemented with 0.1 nM nisin. Incubation was continued for 16 h.

Growth inhibition was measured after 3 h of incubation using microwell plates containing indicator cells at an initial optical density at 600 nm (OD_{600}) of 0.1 and a series of twofold dilutions of the nisin mutant. The 50% inhibitory concentration (IC_{50}) was obtained from the midpoint of the sigmoidal growth curve.

Inhibition of the outgrowth of spores. *Bacillus subtilis* 168 was cultivated at 37°C in Schaeffer sporulation medium (34) in a rotary shaker at 200 rpm for 20 h. The culture was stored at 4°C for one day to lyse. The spores were collected by centrifugation for 10 min at 2,057 × *g* in a swing-out rotor. The loose pellet that contained the spores was extensively washed with Millipore water and stored at 4°C in Millipore water. The spores were heat activated for 10 min at 80°C just prior to use. Equal amounts of spores and cells (5×10^6) were tested in a growth assay in microwell plates as described above. The outgrowth of spores was followed by measuring the OD_{600}. For nisin purified from the supernatant of *L. lactis* NZ9700, the IC_{50} values were found to be identical to the values obtained from nisin A isolated after production by using *L. lactis* containing plasmid-encoded enzymes and plasmid-encoded prepropeptide.

Purification of nisin and nisin mutants. Wild-type nisin A, the ring A KSI, KFI, and VFG mutants, and ring B PT and PH mutants were purified as follows. Cultures were started from an overnight preculture in GM17 medium with antibiotics by diluting them 100-fold into 250 ml minimal medium (32) buffered with 0.12 M MOPS (pH 7.0) buffer, without antibiotics. The supernatant was diluted with an equal volume of 100 mM lactic acid (pH 4), and subsequently, purification proceeded with a single passage of prenisin over a 5-ml fast protein liquid chromatography HiTrap sulfopropyl cation-exchange column (Amersham). Elution was performed at pH 4.0 with 1 M NaCl in 50 mM lactic acid. The fraction containing prenisin was desalted on a PD10 column. The collected desalted fractions were thereafter lyophilized. The leader peptide was completely cleaved off by incubating prenisin with NisP-expressing cells for 16 h. Alternatively, 30 min of incubation at 37°C with 0.01 mg/ml trypsin, which readily cleaves after an arginine, was applied. Mass spectrometric measurement of the high-performance liquid chromatography (HPLC)-purified nisin mutants proved that trypsin had not cleaved after lysines. The nisin and nisin mutants were purified on a C_{18} column with reversed-phase HPLC with a gradient of 10 to 50% acetonitrile in 0.1% trifluoroacetic acid. The purified peptide was quantified on the basis of a comparison of the 214-nm HPLC peak area with that of a nisin A standard of known concentration. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the peptides were >95% pure.

Induction capacity. The induction capacities of the nisin mutants were determined qualitatively by mass spectrometric measurement of the production of the ITRICK leader peptide by the induced *L. lactis* NZ9000 Δ*acmA* containing pTP-ITRICK and pIL3BTC. Quantitative measurement of the induction capacities of the nisin mutants was performed by inducing cells expressing the nisin ring A VFG mutant in the absence of inducing amounts of nisin A. The nisin VFG mutant-expressing cells were incubated for 16 h in GM17 medium containing the appropriate antibiotics with different concentrations of the purified nisin mutant. The trypsin-released activity was determined by measuring growth inhibition, as described above in "Culturing," with the indicator strain *L. lactis* LL108(pOri 280). The lowest concentration needed to get full expression of the VFG mutant was taken as the minimal induction concentration. This bactericidal

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference(s) or source
Strains		
Lactococcus lactis NZ9000	*nisRK*$^+$	23
L. lactis PA1001 (derived from NZ9000)	*nisRK*$^+$ Δ*acmA*	3
L. lactis NZ9700	*nisABTCIPRKEFG*	23
L. lactis LL108(pOri 280)	Em^r Cm^r	23, 26
Micrococcus flavus B423		17
Streptococcus thermophilus ST11	Δ*lacS*	31
Staphylococcus epidermidis K7		37
Bacillus subtilis 168	*fenABCDE* Δ*sfp* Δ*srf*	25
Leuconostoc mesenteroides 20343		DSMZ
Lactobacillus johnsonii 10533		DSMZ
Pediococcus pentosaceus 20336		DSMZ
Plasmids		
pIL253-derived plasmids		35
pIL3BTC	*nisBTC*	32
pNZ8048-derived plasmids		24
pNZnisA-E3	Nisin A	18
pNGnisP	*nisP*	18
pTP-ITRICK	Leader ITRICK	32
pTP-KFI	Encodes I4K S5F L6I nisin A	This study

mutant was used because it has no induction capacity when it is expressed as prenisin in liquid cultures but still has full nisin activity when the leader sequence is cleaved off.

Mass spectrometry. Peptides were isolated from culture supernatants in a single step by applying the ziptip (C18 ziptip; Millipore) procedure as previously described (32) and the zipplate (Millipore) procedures according to the protocol. The dried eluent was resuspended in 5 μl of 50% acetonitrile containing 0.1% (vol/vol) trifluoroacetic acid, and 1 μl was applied to the target. Purified peptides were directly applied to the target. Subsequently, 1 μl of matrix (5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% [vol/vol] trifluoroacetic acid) was added to the target and allowed to dry. The cysteinylation peaks were identified by comparing samples that were either incubated for 15 min at room temperature with 0.05 mg/ml triscarboxyethyl phosphine or not. The thioether ring formation was assessed by an absence of reactivity with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) as described previously (16). Mass spectra were recorded with a Voyager-DE PRO matrix-assisted laser desorption ionization–time-of-flight mass spectrometer (Applied Biosystems). In order to maintain high sensitivity, an external calibration was applied.

Δψ dissipation. The capacity of the nisin mutants and truncated nisin mutants to dissipate the transmembrane electrical potential of *L. lactis* LL108(pOri 280) was measured by using a $DiSC_3(5)$ probe as described previously (13). *L. lactis* (25 μg protein/ml) was suspended in a buffer at 30°C and pH 7.0, prepared by adding piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) to 50 mM NaOH, which buffer contained the Δψ-sensitive probe $DiSC_3(5)$ (6 μM); subsequently, fluorescence was measured at excitation and emission wavelengths of 643 and 666 nm, respectively. By the addition of valinomycin (0.2 μM), a Δψ was induced, resulting in a decrease of the $DiSC_3(5)$ fluorescence. The dissipation of the Δψ resulted in the recovery of the initial level of fluorescence. The fluorescence was measured by using a fluorolog fluorimeter (Jobin Yvon).

RESULTS

Mutagenesis of ring A and B amino acid residues of nisin. Ring A of nisin is composed of a lanthionine that encompasses residues Ile4, Ser5, and Leu6. We simultaneously replaced these residues by randomizing their codons in the *nisA*-carrying plasmid pNZnisA-E3. In a separate experiment, ring B was mutagenized by randomizing the codons for the amino acids in positions 9 and 10 (Fig. 1). We investigated whether the highly conserved (32) Pro9 Gly10 residues are required for the activity of nisin. The plasmid mixtures containing randomized ring A or ring B codons were transformed into *L. lactis* NZ9000 Δ*acmA*, containing plasmid pIL3BTC which encodes the ring-forming enzymes NisB and NisC and the transporter, NisT. This host strain does not contain the leader peptidase, NisP. Hence, the leader peptide remains attached to the produced nisin mutants, thereby keeping them inactive. Following secretion, the leader peptide was removed in vitro by a low concentration of trypsin or by NisP-expressing cells, resulting in identical activity. A large number of transformants was obtained (see Tables S1 and S2 in the supplemental material). About 7% of the randomly tested mutants displayed antibiotic activity against the indicator strain, *L. lactis* LL108(pOri 280). All ring B mutants were also screened qualitatively for their inducing capacities by studying the production of the ITRICK leader peptide. Antimicrobially active mutants and ring B mutants that had inducing capacity were sequenced. Besides wild-type nisin, five mutants were purified for detailed characterization because of particular characteristics as revealed by preliminary experiments: ring A KFI, KSI, and VFG mutants and ring B PT and PH mutants. The KSI mutant was selected for purification because the supernatant of KSI-producing bacteria was about 10 times more potent in inhibiting *L. lactis* LL108(pOri 280) than wild-type nisin A. Because of the high number of phenylalanine-containing mutants and the high antimicrobial activity found for the KSI mutant, an additional, KFI, ring A mutant was made. A particular further reason for constructing and also purifying this KFI mutant was its mimicking of ring A of epidermin. The VFG mutant combined potent antimicrobial activity with an absence of induction capacity in liquid media. By contrast, the ring B PH mutant was purified because of its induction capacity combined with an absence of antimicrobial activity. The PT mutant was the ring B mutant with the most potent antimicrobial activity. These purified mutants were accurately characterized with respect to their antimicrobial activities against different gram-positive bacteria, induction capacities, inhibition of the outgrowth of spores, and capacity to permeabilize the membrane.

A strikingly high number of phenylalanine-containing ring A mutants was obtained (see Table S1 in the supplemental material). Only four of the mutants did not contain a phenylalanine at position 4, 5, or 6 (KSI, TKI, VGG, and YQI). Two mutants harbored a charged residue, i.e., a lysine (KSI and TKI). Most of the mutant residues were hydrophobic. The numbers of variants with three (FFY), two (FVW, FSY, FVS, FSF, FAF, FFS, FFL, FFY, FFV, and SFF), and one (SFV, VFG, IFS, LFQ, LFA, LSF, and YQI) aromatic residue(s) were 1, 10 and 7, respectively. Interestingly, epidermin and gallidermin contain a phenylalanine at position 5, which in nisin corresponds to a serine that is dehydrated during the posttranslational modification. This serine residue was retained in only 10 transformants, while one mutant (TKI) contained a threonine. The extent of dehydration of all mutants was determined by mass spectrometry (see Table S1 in the supplemental material). Among the mutants that did not lack any dehydrated residue were not only very active ones but also mutants with strongly reduced activity. These data indicate the importance of the amino acid composition for the antimicrobial activity.

Despite the high level of conservation of ring B, several active mutants were obtained (see Table S2 in the supplemental material). Mass spectrometric analysis demonstrated that a significant fraction of the SS, PS, TS, and PT mutants contained an extra dehydrated residue compared to those in wild-type nisin. The PH, PR, PD, PN, PL, and PP mutants lacked a dehydrated residue and showed partial Cys addition, indicating that one cysteine had not undergone cyclization via coupling to a dehydroresidue.

Autoinduction and induction. In an overlay assay with sensitive *L. lactis* cells and trypsin, a variety of halo sizes was observed on the plates when nisin was omitted (see Fig. 2, left plate). This implies that even without the inducer nisin, many of the nisin variants were produced and autoinduced. The presence of inducing amounts of nisin in the plates resulted in an increase in halo size (Fig. 2, right plate). When the assay was performed in the absence of trypsin but using NisP-expressing cells for the overlay, the same halo sizes were observed as in the presence of trypsin. Combining trypsin with NisP-expressing cells led neither to larger nor to smaller halo sizes, which suggests that NisP-mediated leader cleavage was complete and demonstrates that the trypsin-mediated removal of leader peptide did not result in inactivation by cleavage in nisin itself. In the absence of trypsin or NisP, no halos were observed except around the wild-type nisin A-producing strain

FIG. 2. Growth inhibition by nisin ring A and B mutants. Two amino acids correspond to positions 9 and 10 of the nisin mutant, and three amino acids correspond to positions 4, 5, and 6 of the nisin mutant. The *L. lactis* Δ*acmA* mutant containing pIL3BTC and the nisin mutant-encoding plasmid was grown overnight on the plates and subsequently covered with a layer of *L. lactis* LL108(pOri 280) and trypsin to cleave off the leader peptide in the presence (right plate) or absence (left plate) of inducing amounts of nisin. Plates were incubated overnight. Halos correspond to zones in which inhibition of the growth of the overlaid strain occurred. In the absence of trypsin, no halos were observed except for the NisP-expressing wild-type strain NZ9700 (wt1 and wt2).

NZ9700, indicating that endogenous leader cleavage is limited. These data indicate that many mutants are equipped with an autoinducing capacity and that all tested mutants could be processed by NisP irrespective of the amino acid composition of ring A and ring B.

In a liquid medium, most leader peptide-containing mutants were unable to self induce. Only prenisin A itself and the ring B PT and PS mutants were able to self induce. Interestingly, after leader cleavage, the ring B PH mutant, which is devoid of antimicrobial activity, also displayed induction capacity (see Table 3). No self-induction was observed when the ring B PH and PR mutants were produced as prenisin. The PR mutant was treated with trypsin and analyzed by mass spectrometry, showing that this nisin mutant is cleaved after the R site (Fig. 3). The N-terminal fragment lacked one dehydrated residue, indicating that Thr at position 8 was not dehydrated. Hence, the observed masses of 1,038, 2,453, and 3,471 Da correspond to IDhbLanIDhaLLanTPR, fragment C11-K34, and the complete nisin PR variant, respectively. Together, these data demonstrate that ring B was not closed, likely because it is not formed in the PR mutant nor, likely, in the PH mutant. Apparently, bulky mutations in position 10 may prevent dehydration at position 8. Hence, it is not only the residues that directly flank the serine or threonine residue that affect dehydration but also residues more remote from these positions. Previously, we observed that ring formation is needed for cleavage by NisP (18). Now, we show that the cleavage reaction does not require a closed ring B. Also, the ring B PD, PN, PL, and PP mutants, which lack one dehydrated residue (see Table S2 in the supplemental material) and concomitantly show only partial Cys addition, were inducers. The presence of induction capacity of the above open-ring-B mutants indicates that ring B is not needed for the activation of NisK. The fully modified prenisin form of the ring A VFG mutant had no capacity to autoinduce in liquid medium, but after cleavage of the leader, the VFG mutant has significant bactericidal activity. Hence the strain that produced this fully modified prenisin form of the ring A VFG mutant could be induced and the induction capacity could be quantified by measuring the bactericidal activity of the produced VFG mutant after leader cleavage. Therefore, the induction capacity in liquid medium of the purified mature forms, without leader peptide, of nisin and its mutants was measured using the strain coding for the VFG mutant. Table 3 shows the minimal concentrations for the variants to obtain maximal induction. The ring B PT mutant was as good an inducer as nisin A, while the induction capacities of the other mutants decreased, with capacities ranging in descending order from KFI to KSI to VFG to PH.

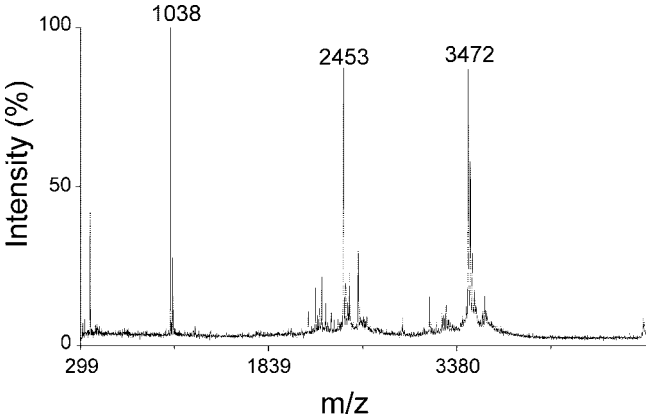

FIG. 3. Trypsin digestion of nisin G10R mutant analyzed by mass spectrometry. The observed average masses correspond to that of IDhbLanIDhaLLanTPR (observed, M + H^+, 1,038 Da; theoretical value of three-times-dehydrated peptide, 1,037 Da): fragment C11-K34, observed, 2,453 Da; theoretical value, 2,454 Da for four-times-dehydrated peptide; whole G10R nisin, observed, 3,471 Da; theoretical value of seven-times-dehydrated peptide, 3,472 Da.

Growth inhibiting activity. The levels of activity of nisin mutants against *L. lactis* and other bacteria were compared

TABLE 2. Susceptibility of cells of different strains to purified nisin and derived ring A and B mutants

Strain	IC_{50} (nM) of[a]:				
	Nisin A	PT mutant	KSI mutant	KFI mutant	VFG mutant
L. lactis NZ9700	>100	>100	**18**	**10**	**2.7**
L. lactis LL108(pOri 280)	0.4	0.75	**0.25**	0.4	1.3
L. lactis LL108(pOri 280)[b]	3.6	58	67	6.1	125
S. epidermidis	2.1	5.2	5.6	3.1	11
S. thermophilus	1.2	2.4	**0.9**	1.5	2.5
P. pentosaceus	0.5	1.5	1.3	**0.4**	2.4
L. johnsonii	0.2	0.30	**0.1**	**0.02**	0.2
L. mesenteroides	0.09	0.19	**0.07**	**0.01**	0.12
M. luteus	0.06	0.20	0.08	**0.03**	0.39

[a] Data are the means of the results of three independent experiments. Values lower than those for nisin A are in bold.

[b] Data are the IC_{50} values of the truncated (Δ23–34) versions of the peptides against *L. lactis* LL108(pOri 280).

with the level of activity of wild-type nisin A (Table 2). The KFI mutant was more active than nisin A against *L. johnsonii* (10-fold-lower IC_{50}) and *L. mesenteroides* (ninefold-lower IC_{50}) (Table 2). The purified KSI mutant (Table 2) showed 1.6- and 2-fold-higher activities than nisin A against indicator strains *L. lactis* LL108(pOri 280) and *L. johnsonii* but was 2.6-fold less active against *S. epidermidis* and *P. pentosaceus*. This mutant contains a conserved mutation, L6I, and a charge alteration, I4K. The TKI mutant has similar amino acids but in a different order, and hardly showed antimicrobial activity. Interestingly, epidermin, gallidermin, and the recently discovered nisin U (42) also contain a lysine at position 4, while epidermin also contains an isoleucine at position 6. These data demonstrate that the strain-specific bactericidal activity of nisin can be enhanced by altering the composition of ring A.

Media containing the ring B TS, SS, AA, or GS mutants showed lower growth-inhibiting activities than medium containing nisin A. Therefore, further mutagenesis was performed by randomizing only position 10 of nisin A, and transformants were analyzed for the production of growth-inhibiting nisin mutants. Mutant PA contained all five thioether rings and showed a modest growth-inhibitory activity (see Table S2 in the supplemental material). The PT mutant, in which the threonine was completely dehydrated, was about 1.5- to 3-fold less active than nisin A (Table 2). These data show that ring B can be composed of other amino acids than P and G but, for antimicrobial activity, small amino acids are required. With the preferred proline at position 9, these amino acids likely favor ring formation.

Nisin mutants that escape the nisin self-protection systems. Striking differences were observed in the comparison of the antimicrobial activities of the mutants when using *L. lactis* NZ9700, a nisin producer, as the indicator strain (Table 2). Ring A VFG (IC_{50}, 2.7 nM), KFI (IC_{50}, 10 nM), and KSI (IC_{50}, 18 nM) mutants showed efficient growth inhibition of *L. lactis* NZ9700 (Table 2), while significant activities were also observed with a range of mutants: in decreasing order, FFS, FVS, IFS, FFS, FFY, FVW, LFA, LFQ, FLI, and FFV (not shown). Apparently these mutants circumvent or inhibit the self-protection mechanisms of NisI and/or NisEFG of strain NZ9700.

Inhibition of the outgrowth of spores. The capacities of the purified nisin A and nisin PT, KSI, KFI, and VFG mutants to inhibit the outgrowth of *Bacillus subtilis* strain168 spores were determined. In parallel, the growth inhibition of *B. subtilis* 168 cells was also measured. The respective IC_{50} values are shown in Fig. 4. While nisin A inhibited the growth of *B. subtilis* 168 cells with an IC_{50} of 7.9 ± 0.6 nM (mean ± standard deviation), much lower concentrations sufficed to inhibit the outgrowth of spores, i.e., an IC_{50} of 0.3 ± 0.2 nM. The tested mutants showed a similar pattern (Fig. 4). Since the KFI and VFG mutants efficiently inhibit the outgrowth of spores, we conclude that Dha5 is not required for this activity. The KFI and KSI mutants even showed an enhanced capacity to inhibit the outgrowth of spores compared to that of nisin A (Fig. 4).

Truncated nisin mutants. To investigate whether the pore formation and lipid II abduction mechanisms can be separated, C-terminally truncated variants of nisin A and its PT, PH, KSI, KFI, and VFG mutants were constructed. Herein, rings D and E were removed, resulting in nisin A(Δ23–34) mutants. The peptides were purified and subjected to mass spectrometry to determine whether rings A, B, and C were correctly formed. In mutant PH(Δ23-34), two thioether bridges were lacking and a disulfide was present instead (see Table S3 in the supplemental material). For the other truncated mutants, the measured masses agreed with the expected masses for the correct formation of all three thioether rings. All cysteine residues were part of lanthionines, as demonstrated by the lack of CDAP addition (see Table S4 in the supplemental material). To investigate whether the bridging patterns of the truncated mutants were

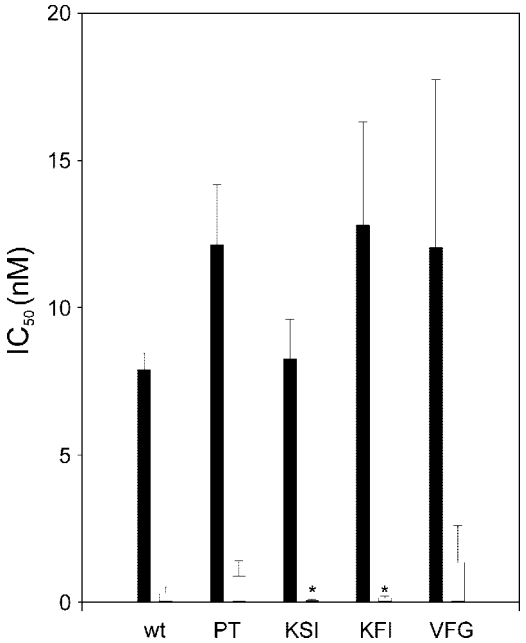

FIG. 4. IC_{50} values for the inhibition of *Bacillus subtilis* 168 cells and spores by nisin A and derived mutants. Cells (filled bars) and spores (open bars) were incubated for 4.5 h with nisin or indicated mutants, after which the OD_{600} was measured. For experimental details, see Materials and Methods. The levels of pore inhibition by nisin KSI and KFI mutants that differed significantly ($P < 0.05$) from that of the wild type (wt) by a paired *t* test are indicated with an asterisk. Error bars indicate standard deviations.

TABLE 3. Induction capacities of full-length and truncated (Δ23–34) nisin A and of ring A and B mutants derived therefrom, all without leader peptide[a]

Peptide	Minimal induction concn (pM)
Full-length peptides	
Nisin A	0.3
PT	0.3
PH	230
KSI	30
KFI	9
VFG	120
Δ23–34 truncated peptides	
Nisin A	40
PT	40
PH	>4,000
KSI	>4,000
KFI	450
VFG	>4,000

[a] *L. lactis* NZ9000 Δ*acmA*(pIL3BTC pTP-nisVFG) was incubated with different concentrations of inducing nisin mutants. The activity of the VFG nisin produced was determined by measuring growth inhibition with the indicator strain *L. lactis* 108(pOri 280). The minimal induction capacity was determined as described in Materials and Methods. The experiment was repeated three times; the values shown are from a typical experiment.

identical to those of full-length mutant peptides, postsource decay was applied and the fragments were analyzed (see Table S5 in the supplemental material). Since only a few fragments were observed, conclusions about the bridging patterns could be drawn. The observation of fragments y10 and y11, which correspond to ring C (Fig. 1) indicates that this ring is correctly formed. The y14 ion is also found for all mutants, indicating that Cys11 of ring B is not connected to the alternative dehydroamino acid Dha5. Thus, rings B and C are correctly formed. With the exception of the PH mutant, fragments that confirm normal bridging patterns were obtained, indicating that ring A is also properly formed (see Table S5 in the supplemental material).

The truncated nisin A and derived mutants showed strongly decreased induction capacities, but truncated nisin A and mutant PT remained more-effective inducers than the other mutants (Table 3). The truncated mutants had retained some antimicrobial activity against *L. lactis* LL108(pOri 280) (Table 2), but this varied among the mutants. Truncated nisin A (IC_{50}, 3.6 nM) and the truncated KFI mutant (IC_{50}, 6.1 nM) showed significant levels of activity: their IC_{50} values were only 9- and 15-fold higher than that of full-length nisin. Truncation led to larger increases for the PT (77-fold increase in IC_{50}), VFG (96-fold increase in IC_{50}), and KSI (268-fold increase in IC_{50}) mutants (Table 2). This relatively larger decrease in activity for the latter mutants might indicate that pore formation by the corresponding full-length mutants might contribute more to the antimicrobial activity than the inhibition of cell wall synthesis. Next, we determined whether the truncated mutants were still capable of permeabilizing the membrane. Therefore, *L. lactis* LL108(pOri 280) cells were suspended in a sodium buffer and a Δψ was induced by adding the potassium ion ionophore valinomycin. The resulting Δψ caused by the electrogenic efflux of potassium ions was monitored by the decrease in the fluorescence of the Δψ-sensitive probe $DiSC_3(5)$. The addition of full-length nisin A (Fig. 5, dotted line) or the KSI, PT, KFI, or VFG mutant (not shown) caused a rapid dissipation of the Δψ, as shown by the recovery of the $DiSC_3(5)$ fluorescence. In contrast, neither truncated nisin A (Fig. 5, solid line) nor any of the other truncated mutants affected the Δψ. Hence, the removal of the C-terminal region containing rings D and E resulted in a complete loss of the membrane permeabilization activity. This implies that the antimicrobial activity observed for the truncated peptides likely results from the remaining capacity to inhibit cell wall synthesis. To validate this suggestion, the ability to inhibit the outgrowth of *B. subtilis* spores was also monitored. This activity was diminished to an extent similar to that of the ability to inhibit the growth of *B. subtilis* cells (not shown), although the outgrowth of spores was inhibited at lower concentrations than those resulting in the inhibition of cells, as discussed before for inhibition by full-length peptides. These data indicate that the inhibition of cell wall synthesis contributes significantly to the antimicrobial effect of nisin A and the KFI mutant but less to the antimicrobial effects of the KSI, VFG, and PT mutants.

DISCUSSION

Following binding to the peptidoglycan precursor lipid II, nisin exhibits two activities that ultimately result in cell death: (i) nisin permeabilizes the membrane by forming transmembrane hybrid pores composed of lipid II and nisin (12, 38) and (ii) it inhibits cell wall synthesis (4, 5, 6) by displacing lipid II

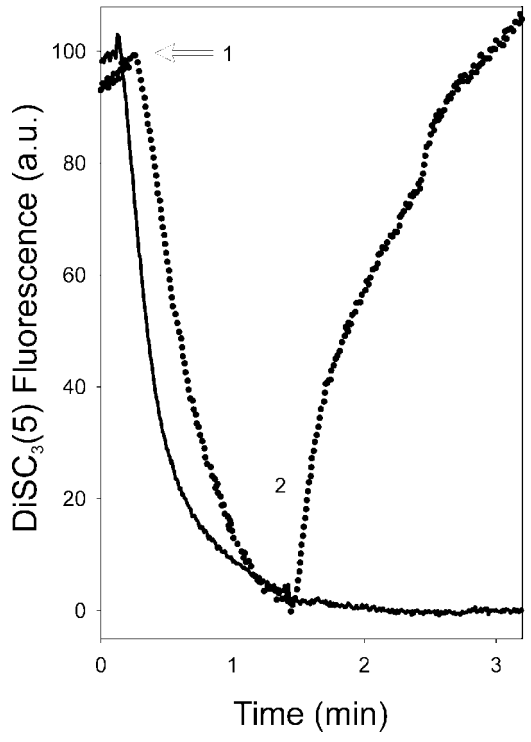

FIG. 5. Nisin A(Δ32–34) is defective in Δψ dissipation in *L. lactis*. *L. lactis* strain LL108(pOri 280) (25 μg protein/ml) was suspended in 50 mM NaOH-PIPES, pH 7.0, at 30°C, containing the Δψ-sensitive probe $DiSC_3(5)$ (6 μM). Arrow 1, addition of valinomycin (0.2 μM); arrow 2, addition of nisin A (3 nM) (dotted line) or nisin A(Δ23–34) (7 nM, solid line). a.u., arbitrary units.

from the septa (11). The binding of nisin to lipid II involves a pyrophosphate cage, formed by rings A and B of nisin (15). Differences at positions 4, 5, and 6 in natural homologs of nisin reside at the circumference of the pyrophosphate cage. This suggested that mutations would be allowed in positions 4 to 6. Nisin also autoinduces its own synthesis by a mechanism involving the interaction with NisK, inhibits the outgrowth of spores, and is recognized by the self-protection mechanisms that provide immunity to the producing cells. The latter involves NisI and the ABC transporter NisEFG.

Here we demonstrate that by mutating ring A or B at one or more positions (i.e., positions 4, 5, and 6, as well as 9 and 10) one or more of nisin's functional characteristics can be modulated. We obtained a series of mutants with entirely new combinations or selections of the activities known for nisin. Mutants with enhanced or reduced bactericidal activity, with strongly reduced antimicrobial activity but significant maintenance of induction capacity, without significant autoinduction capacity but with antimicrobial activity, and with the ability to circumvent the self-protection mechanism of the producing strain were obtained. In addition, mutants that lacked Dha5 but that retained or even showed an enhancement in the capacity to inhibit the outgrowth of spores were obtained. These data clearly indicate that the various activities of nisin can be modulated by altering the composition of ring A or B. The nisin fragment comprising residues 1 to 12 [nisin(1–12)] containing rings A and B does not have antimicrobial activity and has only low induction capacity (10), but as far as antimicrobial activity is concerned, it retains nisin-antagonizing activity (8). Membrane binding by this fragment is strongly reduced (28), possibly because the peptide bears only one positive charge, a lysine. Our data on the ring A VFG mutant indicate that the composition of ring A affects its (auto)inducing capacity. With leader peptide, the VFG mutant had no inducing capacity, and without leader peptide, only poor inducing capacity. However, the VFG mutant retained strong antimicrobial activity. Apparently there is no or no strong correlation between lipid II binding and induction capacity. Changes throughout the nisin molecule affect the signaling capacity, the A ring being essential (10). The C-terminal truncation of residues 23 to 34 described in this report reduced the induction capacity. Charge alterations in the C-terminal part of nisin also resulted in a reduction of induction capacity (40). These data indicate that several regions of the nisin molecule contribute to its induction capacity.

A nisin variant with an open ring C has been reported to have lost essentially all antimicrobial activity (8). Here we report on variants with mutations of rings A, B, and C that are still equipped with antimicrobial activity. Therefore, it seems that ring C is needed for activity, possibly by binding other (truncated) nisin peptides and inducing the segregation of lipid II (11). Nisin A(Δ23–34) and mutants derived therefrom are unable to permeabilize the target membrane but retain antimicrobial activity. This activity of truncated nisin A is about 10-fold higher than that reported for nisin(1–20) (8), which could be the result of the additional positive charge provided by Lys22. The remaining activity of our truncated variants likely results from the inhibition of cell wall synthesis following binding to lipid II. This opens the possibility of selectively optimizing truncated nisin mutants with respect to cell wall synthesis inhibition. The relevance of the mechanism of inhibition of cell wall synthesis has been reinforced by the activity of several highly bactericidal lantibiotics that do not form pores, such as short lantibiotics (2) and a hinge-region mutant of nisin (N20P M21P) (38, 41). Bonelli et al. (2) demonstrated that, in model membranes, lipid II-mediated pore formation by gallidermin, which shares the ring pattern of rings A and B of nisin but consists of only 22 amino acids, depends on the membrane thickness. With intact cells, pore formation was less pronounced than that observed with nisin and occurred only in some strains. Moreover, gallidermin is approximately 10-fold more effective in cell killing than nisin, which might be related to a more-efficient inhibition of cell wall biosynthesis. Ring C, the hinge region, and the C terminus of nisin have been subjected to a limited mutational analysis (8, 22, 39). Interestingly, the N20K and M21K mutants displayed antimicrobial activities against gram-negative *Shigella*, *Pseudomonas*, and *Salmonella* species (43). Further mutational analysis of these regions may result in additional mutants with interesting characteristics.

Nisin has been reported to inhibit the outgrowth of spores via a mechanism that involves Dha5, which is thought to react with protein thiol groups in the spore wall. Dha5 is indeed a reactive residue and likely the least-stable residue of ring A that is of functional importance. Here we obtained nisin mutants without a Dha residue in ring A that efficiently inhibit the outgrowth of spores. In addition, C-terminal truncation of nisin A, while the Dha5 was retained, resulted in a major loss in the spore outgrowth inhibitory activity. Therefore, our data demonstrate that this residue is not essential for spore outgrowth inhibition. This opens the possibility of generating new nisin variants, devoid of any unstable dehydroresidue, which still effectively inhibit the outgrowth of spores.

Previously, we have shown that the lantibiotic enzymes have a broad substrate specificity (16, 18, 32). Now we demonstrate that the activities of nisin can be modulated differentially by mutagenesis of rings A and B. Already, when selecting only against *L. lactis*, a large number of interesting ring A and B mutants were obtained. These rings show a large mutational freedom and can be used as targets for improving lantibiotics. Therefore, it will be of interest to apply the same method for randomization of rings A and B and select for activity against pathogenic strains. In combination with the possibility of engineering new lanthionine rings, tremendously large possibilities exist for the design and synthesis of new lantibiotics and bioactive peptides with desired properties.

ACKNOWLEDGMENTS

G. Bierbaum is gratefully acknowledged for *S. epidermidis* K7 strain.

L. D. Kluskens is supported by STW grant 6927 and R. Rink and A. Kuipers by the Province of Groningen, grant IAG B09.

REFERENCES

1. **Bierbaum, G.** 1999. Genetics of lantibiotic biosynthesis in amino acids, peptides, porphyrins, and alkaloids, p. 275–301. *In* D. Barton, K. Nakanishi, and O. Meth-Cohn (ed.), Comprehensive natural products chemistry. Elsevier, Amsterdam, The Netherlands.
2. **Bonelli, R. R., T. Schneider, H.-G. Sahl, and I. Wiedemann.** 2006. Insights into in vivo activities of lantibiotics from gallidermin and epidermin mode-of-action studies. Antimicrob. Agents Chemother. **50:**1449–1457.
3. **Bosma, T., R. Kanninga, J. Neef, S. A. Audouy, M. L. van Roosmalen, A. Steen, G. Buist, J. Kok, O. P. Kuipers, G. Robillard, and K. Leenhouts.** 2006. Novel surface display system for proteins on nongenetically modified gram-positive bacteria. Appl. Environ. Microbiol. **72:**880–889.

4. **Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H.-G. Sahl, and B. de Kruijff.** 1999. Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science **286:**2245–2247.
5. **Breukink, E., H. E. van Heusden, P. J. Vollmerhaus, E. Swiezewska, L. Brunner, S. Walker, A. J. R. Heck, and B. de Kruijff.** 2003. Lipid II is an intrinsic component of the pore induced by nisin in bacterial membranes. J. Biol. Chem. **278:**19898–19903.
6. **Brötz, H., M. Josten, I. Wiedemann, U. Schneider, F. Götz, G. Bierbaum, and H.-G. Sahl.** 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol. Microbiol. **30:**317–327.
7. **Chan, W. C., H. M. Dodd, N. Horn, K. Maclean, L. Y. Lian, B. W. Bycroft, M. J. Gasson, and G. C. Roberts.** 1996. Structure-activity relationships in the peptide antibiotic nisin: role of dehydroalanine 5. Appl. Environ. Microbiol. **62:**2966–2969.
8. **Chan, W. C., M. Leyland, J. Clark, H. M. Dodd, L.-Y. Lian, M. J. Gasson, B. W. Bycroft, and G. C. K. Roberts.** 1996. Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin. FEBS Lett. **390:**129–132.
9. **Chatterjee, C., M. Paul, L. Xie, and W. A. van der Donk.** 2005. Biosynthesis and mode of action of lantibiotics. Chem. Rev. **105:**633–684.
10. **Dodd, H. M., N. Horn, W. C. Chan, C. J. Giffard, B. W. Bycroft, G. C. K. Roberts, and M. J. Gasson.** 1996. Molecular analysis of the regulation of nisin immunity. Microbiology **142:**2385–2392.
11. **Hasper, H. E., N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff, and E. Breukink.** 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science **313:**1636–1637.
12. **Hasper, H. E., B. de Kruijff, and E. Breukink.** 2004. Assembly and stability of nisin-lipid II pores. Biochemistry **43:**11567–11575.
13. **Hauge, H. H., D. Mantzilas, G. N. Moll, W. N. Konings, A. J. Driessen, V. G. Eijsink, and J. Nissen-Meyer.** 1998. Plantaricin A is an amphiphilic alpha-helical bacteriocin-like pheromone which exerts antimicrobial and pheromone activities through different mechanisms. Biochemistry **37:**16026–16032.
14. **Holo, H., and I. F. Nes.** 1995. Transformation of Lactococcus by electroporation. Methods Mol. Biol. **47:**195–199.
15. **Hsu, S.-T. D., E. Breukink, E. Tischenko, M. A. G. Lutters, B. de Kruijff, R. Kaptein, A. M. J. J. Bonvin, and N. A. J. van Nuland.** 2004. The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nat. Struct. Mol. Biol. **11:**963–967.
16. **Kluskens, L. D., A. Kuipers, R. Rink, E. de Boef, S. Fekken, A. J. M. Driessen, O. P. Kuipers, and G. N. Moll.** 2005. Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. Biochemistry **44:**12827–12834.
17. **Kramer, N. E., E. J. Smid, J. Kok, B. de Kruijff, O. P. Kuipers, and E. Breukink.** 2004. Resistance of Gram-positive bacteria to nisin is not determined by lipid II levels. FEMS Microbiol. Lett. **239:**157–161.
18. **Kuipers, A., E. de Boef, R. Rink, S. Fekken, L. D. Kluskens, A. J. M. Driessen, K. Leenhouts, O. P. Kuipers, and G. N. Moll.** 2004. NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. J. Biol. Chem. **279:**22176–22182.
19. **Kuipers, O. P., M. M. Beerthuyzen, P. G. G. A. de Ruyter, E. J. Luesink, and W. M. de Vos.** 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J. Biol. Chem. **270:**27299–27304.
20. **Kuipers, O. P., M. M. Beerthuizen, R. J. Siezen, and W. M. de Vos.** 1993. Characterization of the nisin gene cluster nisABTCIPR of *Lactococcus lactis*. Requirement of expression of the nisA and nisI genes for development of immunity. Eur. J. Biochem. **216:**281–291.
21. **Kuipers, O. P., G. Bierbaum, B. Ottenwälder, H. M. Dodd, N. Horn, J. Metzger, T. Kupke, V. Gnau, R. Bongers, P. van den Bogaard, H. Kosters, H. S. Rollema, W. M. de Vos, R. J. Siezen, G. Jung, F. Götz, H.-G. Sahl, and M. J. Gasson.** 1996. Protein engineering of lantibiotics. Antonie Leeuwenhoek **69:**161–170.
22. **Kuipers, O. P., H. S. Rollema, W. M. Yap, H. J. Boot, R. J. Siezen, and W. M. de Vos.** 1992. Engineering dehydrated amino acid residues in the antimicrobial peptide nisin. J. Biol. Chem. **267:**24340–24346.
23. **Kuipers, O. P., P. G. de Ruyter, M. Kleerebezem, and W. M. de Vos.** 1997. Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol. **15:**135–140.
24. **Kuipers, O. P., P. G. de Ruyter, M. Kleerebezem, and W. M. de Vos.** 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. **64:**15–21.
25. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium Bacillus subtilis. Nature **390:**249–256.
26. **Leenhouts, K., A. Bolhuis, G. Venema, and J. Kok.** 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Appl. Microbiol. Biotechnol. **49:**417–423.
27. **Liu, W., and J. N. Hansen.** 1992. Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. J. Biol. Chem. **267:**25078–25085.
28. **Moll, G. N., J. Clark, W. C. Chan, B. W. Bycroft, G. C. Roberts, W. N. Konings, and A. J. M. Driessen.** 1997. Role of transmembrane pH gradient and membrane binding in nisin pore formation. J. Bacteriol. **179:**135–140.
29. **Morris, S. L., R. C. Walsh, and J. N. Hansen.** 1984. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. J. Biol. Chem. **259:**13590–13594.
30. **Otto, M., R. Süsmuth, C. Vuong. G. Jung, and F. Götz.** 1999. Inhibition of virulence factor expression in *Staphylococcus aureus* by the *Staphylococcus epidermidis* agr pheromone and derivatives. FEBS Lett. **450:**257–262.
31. **Poolman, B., J. Knol, B. Mollet, B. Nieuwenhuis, and G. Sulter.** 1995. Regulation of bacterial sugar-H+ symport by phosphoenolpyruvate-dependent enzyme I/HPr-mediated phosphorylation. Proc. Natl. Acad. Sci. USA **92:**778–782.
32. **Rink, R., A. Kuipers, E. de Boef, K. J. Leenhouts, A. J. M. Driessen, G. N. Moll, and O. P. Kuipers.** 2005. Lantibiotic structures as guidelines for the design of peptides that can be modified by lantibiotic enzymes. Biochemistry **44:**8873–8882.
33. **Rollema, H. S., J. W. Metzger, P. Both, O. P. Kuipers, and R. J. Siezen.** 1996. Structure and biological activity of chemically modified nisin A species. Eur. J. Biochem. **241:**716–722.
34. **Schaeffer, P., J. Millet, and J.-P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
35. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70:**559–566.
36. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:**807–813.
37. **van de Kamp, M., H. W. van den Hooven, R. N. Konings, G. Bierbaum, H.-G. Sahl, O. P. Kuipers, R. J. Siezen, W. M. de Vos, C. W. Hilbers, and F. J. van de Ven.** 1995. Elucidation of the primary structure of the lantibiotic epilancin K7 from *Staphylococcus epidermidis* K7. Cloning and characterisation of the epilancin-K7-encoding gene and NMR analysis of mature epilancin K7. Eur. J. Biochem. **230:**587–600.
38. **van Heusden, H. E., B. de Kruijff, and E. Breukink.** 2002. Lipid II induces a transmembrane orientation of the pore-forming peptide lantibiotic nisin. Biochemistry **41:**12171–12178.
39. **van Kraaij, C., E. Breukink, H. S. Rollema, R. S. Bongers, H. A. Kosters, B. de Kruijff, and O. P. Kuipers.** 2000. Engineering a disulfide bond and free thiols in the lantibiotic nisin Z. Eur. J. Biochem. **267:**901–909.
40. **van Kraaij, C., E. Breukink, H. S. Rollema, R. J. Siezen, R. A. Demel, B. de Kruijff, and O. P. Kuipers.** 1997. Influence of charge differences in the C-terminal part of nisin on antimicrobial activity and signaling capacity. Eur. J. Biochem. **247:**114–120.
41. **Wiedemann, I., E. Breukink, C. van Kraaij, O. P. Kuipers, G. Bierbaum, B. de Kruijff, and H.-G. Sahl.** 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. **276:**1772–1779.
42. **Wirawan, R. E., N. A. Klesse, R. W. Jack, and J. R. Tagg.** 2006. Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. Appl. Environ. Microbiol. **72:**1148–1156.
43. **Yuan, J., Z. Z. Zhang, X. Z. Chen, W. Yang, and L. D. Huan.** 2004. Site-directed mutagenesis of the hinge region of nisinZ and properties of nisinZ mutants. Appl. Microbiol. Biotechnol. **64:**806–815.

Supplemental data

DISSECTION AND MODULATION OF THE FOUR DISTINCT ACTIVITIES OF NISIN BY MUTAGENESIS OF RINGS A AND B AND BY C-TERMINAL TRUNCATION

By Rick Rink, Anneke Kuipers, Leon D. Kluskens, Arnold J.M. Driessen, Oscar P. Kuipers and Gert N. Moll

Table S1. Ring A mutants. Concentrations in medium relative to nisin have been determined by a peptide assay according to Bradford.

Positions 4, 5, 6 before dehydration	IC_{50} of mutant nisin against *L. lactis* LL 108 (pOri 280) relative to wild-type nisin A	Dehydrations	
		Observed	Lacking[1]
ISL (wild type)	1	8	0
KSI	0.2	8	0
FSI	2	8	0
TKI	43	7	0
VGG	270	7 (6)	0 (1)[#]

FVW	10	7 (6)	0 (1)[#]
FFL	16	7 (6)	0 (1)[#]
YQI	28	7 (6)	0 (1)[#]
FLI*,	75	7 (6)	0 (1)[#]
LFQ	12	7 (6)	0 (1)[#]
YQI	75	7	0
FSY	5	8, 7, (6)	0, 1, (2)
FVS	5	8, 7	0, 1
FSF	3	(8), 7	(0), 1
FAF	6	7, (6)	0, (1)
FFS	9	8, 7	0, 1
FFV*	15	7	0
SFF	6	(8), 7, (6)	(0), 1, (2)
SFV	10	8, 7	0, 1
VFG	2	7	0
IFS	6	7, (6)	1, (2)
LFA	6	7, 6	0, 1
LSF	19	8, 7	0, 1

* Small peak of +119 Da observed indicating cysteine addition and hence a partly open ring.

[1]Considering Ser33 never being dehydrated. [#]Dehydration values based on small mass spectrometric peaks are given between brackets.

Table S2. Growth inhibiting activity of ring B mutants of nisin. Concentrations in medium relative to nisin have been determined by a peptide assay according to Bradford.

Ring B mutant Positions 9, 10 before dehydration	IC_{50} of mutant nisin against *L. lactis* LL 108 (pOri 280) relative to wild-type nisin A	Dehydrations	
		Observed	Lacking
GC	> 500	ND	ND
GS	17	8	1
SS	13	8, 9	2, 1
AA	23	ND	ND
PH, PR, PD, PN, PL, PP	> 500	7	1
TS	15	9	1
PA	13	8	0
PS	4	8, 9	1, 0
PT	0.9	9	0
PG (wild type)	1	8	0

Table S3. Truncated PH nisin lacks 2 thioether rings and contains a single disulfide bridge. Calculated masses are indicated between brackets and in italics. For the full length version of PH nisin A (Δ23-34), ring B was found not to be closed. To investigate if the rings are formed in the PH nisin A (Δ23-34) truncated variant (Table S3), masses were determined. The mass in the presence of TCEP was 2 Da higher than without TCEP, and 2 Da higher than expected for 3 closed thioether rings A, B and C. CDAP treatment resulted in 50 Da increase in mass. This implies that the two rings of the truncated PH mutant were not formed, and instead a disulfide bond exists under oxidizing conditions. TCEP treatment opens the disulfide bond leading to a 2 Da increase. CDAP treatment corresponds to two additions, i.e., 25 Da per available thiol.

	Monoisotopic mass Da [M + H^+]		
Treatment	none	TCEP	CDAP
PH (Δ23-34)	2235.15 (*2237.05*)	2237.19 (*2237.05*)	2286.86 (*2287.05*)

Table S4. Characterization of truncated nisin and nisin rings A and B mutants by mass spectrometry.

	Monoisotopic mass Da [M + H^+]		Dehydrations
	Observed	Theoretical	
nisin A (Δ23-34)	2139.31	*2139.00*	5
PT (Δ23-34)	2164.97	*2165.02*	6
KSI (Δ23-34)	2154.12	*2154.01*	5
KFI (Δ23-34)	2232.12	*2232.07*	4
VFG (Δ23-34)	2146.97	*2146.97*	4

Table S5. Characterization of truncated nisin mutants by postsource decay.

Mutants	PSD fragment	peptide	Mass Da [M + H^+]	
			Measured	Theoretical
Nisin A (Δ23-34) KSI (Δ23-34) KFI (Δ23-34) VFG (Δ23-34) PT (Δ23-34)	y10	dhbGALMGCNMK	1009	1007 Da
Nisin A (Δ23-34) KSI (Δ23-34) KFI (Δ23-34) VFG (Δ23-34) PT (Δ23-34)	y11	KdhbGALMGCNMK	1136	1135 Da
Nisin A (Δ23-34) KSI (Δ23-34) KFI (Δ23-34) VFG (Δ23-34)	y14	PGCKdhbGALMGCNMK	1395	1393 Da
PT (Δ23-34)	y14	PdhbCKdhbGALMGCNMK	1422	1419 Da